Kallikrein Messenger RNA in Rat Arteries and Veins

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Glandular kallikrein (EC 3.4.21.8) belongs to a subgroup of serine proteases coded by a multigene family. A kininogenase resembling glandular kallikrein has been identified in vascular tissue; however, it is not clear whether it is synthesized by vascular tissue or taken up from plasma. To determine the potential for kallikrein synthesis in vascular tissues, we tested whether messenger RNA (mRNA) for glandular kallikrein is present in rat arteries and veins. Poly(A⁺) RNA was isolated from pools of arteries or veins (n = 3, 30 rats each). Poly(A⁺) RNA from the kidney and liver was used as a positive and negative control, respectively. As a probe, we used rat pancreatic kallikrein ³²P-labeled complementary DNA, which recognizes mRNA of the entire rat kallikrein family. Slot-blot analysis indicated that kallikrein mRNA was present in mRNA from the arteries, veins, and kidney but not from the liver. Poly(A⁺) RNA from arteries and veins contained ~1% as much kallikrein mRNA as that from the kidney. To confirm the slot-blot results and determine whether the mRNA for true glandular kallikrein was present in vascular tissue, we employed a polymerase chain reaction assay, first using primers specific for the entire kallikrein family (which amplify a 430-bp fragment) and then using primers specific for true glandular kallikrein mRNA (which amplify a 370-bp fragment). After the polymerase chain reaction assay, both arteries and veins showed fragments of these sizes when tested with rat kallikrein complementary DNA probe, thus confirming the presence of glandular kallikrein mRNA. Similar results were obtained when the polymerase chain reaction assay was applied to mRNA isolated from vascular smooth muscle cells in culture and from the kidney. No signal was obtained with liver mRNA. We concluded that kallikrein is synthesized by the vascular wall, possibly by smooth muscle cells. The presence of locally synthesized kallikrein indicates that the vascular kallikrein-kinin system may play a role in the regulation of vascular tone. (Circulation Research 1990;67:510–516)

Tissue or glandular kallikrein is a serine protease of restricted substrate specificity that releases kinins (kininogenase activity) from kininogen substrates.¹ Kinins are potent vasodilators both in vivo and in vitro. This vasorelaxant effect has been linked to the ability of kinins to release endothelium-derived relaxing factor and prostacyclins from endothelial cells.²⁻⁵ Kinins also induce venous contraction and increase capillary permeability and lymphatic flow.⁶⁻⁸ There is evidence that kinins play a role in local regulation of blood flow.⁹⁻¹² They have also been implicated as partial mediators of the acute antihypertensive effect of converting enzyme inhibitors.¹³⁻¹⁷ Since the concentration of kinins in the blood either before or after converting enzyme inhibition is below that needed to alter blood pressure,¹⁵,¹⁸,¹⁹ it has been suggested that kinins act as paracrine rather than circulating hormones.²⁰ A kininogenase resembling glandular kallikrein has been found in rat arteries and veins;²¹ however, its origin is not clear, as it may either be synthesized by the vasculature or be extracted from plasma, which contains glandular kallikrein.²² We questioned whether kallikrein messenger RNA (mRNA) is present in rat arteries and veins (indicating kallikrein synthesis) or cultured vascular smooth muscle cells. We isolated mRNA from vascular tissue and demonstrated the presence of kallikrein mRNA by two different assays.

Materials and Methods

Dissection of Tissues and Cell Culture

In each of three separate experiments, 30 Sprague-Dawley rats (300–350 g) were decapitated, the vessels were rapidly dissected and cleaned of perivascu-
lar fat and adventitia, and the arteries (thoracic and abdominal aorta and tail arteries) and veins (superior and inferior vena cava and tail vein) were pooled. The kidneys and liver were also removed. All tissues were immediately frozen and kept in liquid nitrogen until needed.

A smooth muscle cell line (A7r5) from the embryonic thoracic aorta of a DB1X rat was obtained from American Type Culture Collection, Rockville, Md. Cells were grown in culture dishes 100 mm in diameter using Dulbecco’s Modified Eagle Medium supplemented with 10% fetal bovine serum in an atmosphere of 95% O₂, 5% CO₂. At the time of cell harvest, the medium was thoroughly aspirated and cells were immediately lysed by directly adding 15 ml of 4 M guanidine thiocyanate buffer to the dishes to prepare the lysate for RNA extraction.

Total and Poly(A⁺) RNA Extraction

Total RNA was isolated from various tissues or cells in culture by the single-step method.23 Briefly, frozen tissues were homogenized to a fine powder with a mortar and pestle under liquid nitrogen. Aliquots of tissue (1 g) were mixed with 15 ml buffer D containing 4 M guanidine thiocyanate and 25 mM sodium citrate (pH 7.0) with 0.5% sarcosine and 0.1 M 2-mercaptoethanol. After homogenization for 60 seconds with a polytron (Brinkmann Instruments, Inc., Westbury, N.Y.), the homogenate was mixed with 0.1 vol of 2 M sodium acetate (pH 4.0), 1 vol phenol saturated with water, and 0.2 vol chloroform. Samples were centrifuged at 10,000g for 20 minutes at 4°C. The aqueous phase, which contained RNA, was transferred to a new tube and total RNA was precipitated with an equal volume of isopropanol, washed extensively with 70% ethanol, and resuspended in water treated with diethyl pyrocarbonate. Poly(A⁺) RNA was extracted from total RNA by affinity chromatography on oligo (dT)-cellulose.24 The integrity of the isolated RNA was assessed by 1.5% agarose gel electrophoresis; it was stained with ethidium bromide and illuminated with ultraviolet light at 254 nm. The intensity ratio of the 28S:18S ribosomal RNA bands was 2:1, which was taken as an indication of RNA integrity.

Kallikrein Complementary DNA Probe

The recombinant plasmid pcXP39, bearing a rat pancreatic kallikrein complementary DNA (cDNA) insert, was prepared as described previously.25 The probe sequences contained the 3' 550-bp region of rat pancreatic kallikrein mRNA, which encodes the carboxy terminal 167 amino acids of kallikrein plus the 3' untranslated region. For the hybridization studies, the plasmid was digested with HindIII (Boehringer Mannheim Corp., Indianapolis, Ind.) and subjected to electrophoresis on a low-melting-point agarose gel; the kallikrein insert band was extracted from the gel and radiolabeled with ³²P using the random primer method.26

Kallikrein RNA Analysis

Northern and slot blotting were performed as described previously.27,28 For the Northern blot, RNA was denatured in 1 M glyoxal and 50% dimethyl sulfoxide, subjected to electrophoresis in a 1.2% agarose horizontal gel with 10 mM sodium phosphate buffer (pH 7.0), and then transferred by capillary blotting to a Nytran nylon membrane (Schleicher & Schuell, Inc., Keene, N.H.). For the slot blot, RNA was denatured in 1 M glyoxal and then spotted onto Nytran in a slot-format blotting apparatus (Bio-Rad Laboratories, Cambridge, Mass.). The membrane was baked for 2 hours at 80°C in vacuo, prehybridized for 6 hours at 55°C in a solution of 50% formamide, ×5 standard saline citrate (SSC) (×1 SSC is 0.15 M sodium chloride and 0.015 M sodium citrate, pH 7.4; ×5 denotes that the concentration used is five times higher), 50 mM sodium phosphate (pH 7.0), 0.1% sodium dodecyl sulfate (SDS), 0.1% sodium pyrophosphate, ×10 Denhardt’s solution (0.2% bovine serum albumin, Ficoll, and polyvinylpyrrolidone), and 100 µg/ml denatured herring sperm DNA, and then hybridized in a fresh solution containing a ³²P-labeled kallikrein cDNA probe for 20–25 hours. Blots were washed in ×2 SSC, 0.5% SDS twice for 15 minutes each at room temperature, then in 0.1% SSC, 0.5% SDS four times for 15 minutes each at 65°C, and finally in 0.1% SSC twice for 15 minutes each at 65°C. The membrane was then exposed to Kodak X-AR film (Eastman Kodak, Rochester, N.Y.) with a Cornex Lighting Plus intensifying screen (Du Pont Co., Wilmington, Del.) at −70°C for 1–4 days.

Oligonucleotides Used for Amplification of Kallikrein mRNA

Two sets of primers were prepared. The oligonucleotide sequences given below for true kallikrein mRNA were derived from the previously reported kallikrein (PS-type) mRNA sequence.29 These PS-specific primers differ from the other known kallikrein family members at 5–12 positions within the 21 nucleotide primers. The primers for the kallikrein

![FIGURE 1. Schematic diagram of kallikrein gene sequencing.](image-url)
family were derived from oligonucleotide regions conserved among all nine rat kallikrein genes sequenced to date (Figure 1). The oligonucleotides were synthesized by phosphoramidite chemistry on an automated DNA synthesizer (model 380 B, Applied Biosystems, Inc., Foster City, Calif.).

The kallikrein gene family primers are as follows: 1) downstream primer designated as KALPCRU4 (5'-GATGTTCAACACTGGAGATC-3') and 2) upstream primer designated as KALPCRU2 (5'-GATTCCAAACCTGGAAGT-3').

The true kallikrein (PS) primers are as follows: 1) downstream primer designated as PSPCRex3/4 (5'-TCCAATCCGTCAGGTGTGATG-3') and 2) upstream primer designated as PSPCRex2 (5'-TACTACCTGGCGGAAATACCTA-3').

Amplification of kallikrein mRNA using the kallikrein gene family primers should give a 430-bp fragment; however, the true kallikrein primers will give a 370-bp fragment.

Amplification Method

cDNA was synthesized using cloned Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories, Gaithersburg, Md.) according to the manufacturer's protocol and as previously described. The 20-μl reaction mixture contained the enzyme buffer supplied by Bethesda Research Laboratories, together with 1 μg total RNA from RNA samples pretreated with RNase-free DNase (RQ1, Promega Biotec, Madison, Wis.), 1 unit RNasin/μl (Promega Biotec), 10 pmol downstream polymerase chain reaction assay (PCR) primer, 1 mM (each) deoxynucleoside triphosphate, and 200 units reverse transcriptase. The reaction mixture was incubated for 45 minutes at 42°C, heated at 95°C for 5 minutes to kill the enzyme, and chilled on ice. It was then diluted with 80 μl PCR reaction buffer (50 mM KCl/10 mM Tris chloride/1.5 mM MgCl2/100 μg gelatin/ml, pH 8.3), followed by the addition of 10 pmol upstream primer and 1 unit thermostable DNA polymerase from Thermus aquaticus (Taq polymerase). One hundred milliliters of mineral oil was added to prevent evaporation. Control samples were processed exactly as above except that reverse transcriptase was not included in the reaction mixture. The reaction was initiated by heat denaturation of RNA-cDNA hybrid (95°C) for 1 minute and by annealing the primers for 2 minutes at 55°C and then extending them for 3 minutes at 72°C. The cycle was repeated 30 times using a programmable PCR instrument (Perkin-Elmer Cetus Instruments, Norwalk, Conn.). After the final cycle, the temperature was held at 72°C for 10 minutes to help ensure complete elongation.

Analysis of PCR-Amplified Products by Southern Blot

Southern blotting was performed as described previously. We applied 50 μl from each PCR-amplified fraction to 1.5% agarose gel and performed electrophoresis at 100 V for 3–4 hours. The gel was briefly rinsed in deionized water, soaked in 1.0 M NaCl/0.5 M NaOH twice for 15 minutes each with shaking, and then neutralized by soaking in 0.5 M Tris (pH 7.4)/1.5 M NaCl twice for 15 minutes each. The gel was transferred to a Nytran membrane by capillary blotting, after which the membrane was first prehybridized and then hybridized using the kallikrein cDNA probe as described for the Northern blot.

Results

Figure 2 shows the results obtained from the slot-blot analysis. The rat kallikrein cDNA probe hybridized to poly(A+) RNA from the kidney, arteries, and veins but not from the liver. The hybridization signal obtained with 20 μg poly(A+) RNA from the arteries and veins was slightly more intense than that observed with 0.2 μg kidney poly(A+) RNA. This concentration was below the limits of sensitivity of Northern blot analysis, because no signal was obtained with either 20 μg poly(A+) RNA from arteries and veins or 0.2 μg kidney poly(A+) RNA. A clear signal was obtained with 5 μg kidney poly(A+) RNA, for an RNA of 0.90 kilobase (Figure 3).
whereas no amplification was observed with liver mRNA (Figure 4). Similar results were obtained with kidney mRNA (data not shown). When reverse transcription was omitted to eliminate the possibility that primers were amplifying contaminating genomic DNA, no signal was observed (Figure 4). Moreover, due to the presence of introns, amplification of genomic kallikrein should yield fragments approximately 1 kilobase larger.

**Discussion**

A glandular kallikrein-like enzyme has been found in vascular tissue.\(^2\) This enzyme could have originated from either plasma uptake or local synthesis by vascular tissues. The slot-blot assay demonstrated that in rats, kallikrein mRNA is present in both arteries and veins, a finding accepted as an indicator of synthesis.\(^3\) The level of kallikrein mRNA in the kidney has been estimated to be 0.1–0.2% of the mRNA population.\(^4\) Judging from the intensity of hybridization, the level of kallikrein mRNA in the arteries and veins is about 1/30 that of the kidney, or approximately 0.002% of total mRNA. While the specificity of the slot-blot assay has been questioned, we used stringent assay conditions. As expected, mRNA for kallikrein was not detectable in the liver; in contrast, a strong signal was observed with kidney mRNA.\(^5\) Thus, the use of adequate positive and negative controls ensures that the cDNA probe did specifically hybridize to mRNA for one or more species of kallikrein mRNA. Therefore, at least part of the kinin-generating activity detected in vascular tissue may be due to local production by components of the vascular wall.

The kallikrein gene family comprises a set of very closely related genes named after glandular kallikrein.\(^6\) The rat has eight to 17 kallikrein genes,\(^7\)\(^8\) compared with 24 in the mouse\(^9\) and possibly only three or four in humans.\(^9\)\(^10\) Three rat kallikrein cDNA probe used would hybridize to most, if not all, mRNA transcribed from the kallikrein gene family. Thus, the slot-blot results alone do not prove that mRNA for true glandular kallikrein is present in vascular tissue. Furthermore, since the concentration of vascular kininogenase is low compared with organs such as the submandibular gland, pancreas, or kidney,\(^11\) it was not surprising that kallikrein mRNA in arteries and veins was only a fraction of that present in our positive control, the kidney. Its concentration was too low to be detected by our Northern blot analysis, as indicated by the absence of a signal when an equivalent concentration of kidney mRNA was tested.

To confirm the slot-blot results, to solve the problem of sensitivity, and to clarify whether mRNA for true kallikrein is present in vascular tissue, we used the PCR assay, a powerful technique used to amplify low levels of DNA as well as mRNA after conversion to cDNA. PCR can reveal even low amounts of a particular species of mRNA, provided that specific oligonucleotide primers are available; in addition, it
can discern which members of a particular gene subgroup are being expressed. With primers that corresponded to regions of mRNA common to all kallikrein species, a fragment of the predicted size was found, thus confirming the results obtained by slot-blot analysis of arteries and veins. However, we were interested in determining whether mRNA for true glandular kallikrein was present in the tissues we examined, since the ability to interact with kininogen and efficiently release kinins is a characteristic of this enzyme. Other members of the rat kallikrein family, such as tonin, esterase-B, and esterase-γ, either have a different substrate specificity from true glandular kallikrein or else are poor kininogenases. Using mRNA-specific oligonucleotide primers, we found that the vascular tissue most likely contained true kallikrein mRNA. The presence of true kallikrein mRNA cannot be assured rigorously, because the PCR primers are only mRNA-specific relative to the rat kallikrein genes that have been sequenced to date. Consequently, the presence of one or more of the remaining uncharacterized members (estimated between 8 and 11) that may be identical to true kallikrein in the primer regions cannot be excluded. Moreover, the data do not exclude the expression of another kallikrein gene in addition to true kallikrein. Proof of their identity requires sequence analysis of cDNA cloned from vascular tissue RNA. False-positive results have been reported with PCR, which is an extremely sensitive technique. We included the kidney as a positive control and the liver as a negative control; moreover, in every case we ensured that contamination of genomic DNA was not being amplified by treating RNA samples with RNase-free DNase and also by running the PCR without the reverse transcriptase step. The kidney is known to actively synthesize true kallikrein, and the molecular weight of the fragments amplified from the kidney, arteries, veins, and vascular smooth muscle cells in culture was the same. No signal was observed in the samples without reverse transcriptase or with liver mRNA, confirming that the data were not caused by an artifact.

Although it has been reported that neither the cDNA probe nor the oligonucleotides used in this study detect a signal when tested with liver mRNA, these data were not obtained by PCR. The lack of amplification observed with liver mRNA after PCR was not completely predictable. Two thirds of the liver's nourished circulation is portal in origin, and one third is derived from the hepatic artery. The predominant vessels are capillaries lined by endothelial cells and terminal portal vessels that have no smooth muscle fibers. Arterioles join the terminal portal venules in the acinus. Thus, we expected some sort of signal from the liver after PCR. We do not know whether kallikrein mRNA is present in vascular smooth muscle, endothelium, or other vascular...
cells. The fact that we found mRNA for kallikrein in vascular smooth muscle cells in culture suggests that it is at least partially responsible for the mRNA found in arteries and veins. It may be that kallikrein mRNA from vascular smooth muscle cells of the liver accounts for such a small proportion of total mRNA that PCR combined with Southern blotting could not detect it. A second possibility is that the liver mRNA was partially degraded. Although we did not observe any major differences in integrity among the different tissue mRNAs, since the content of kallikrein mRNA must be small, even partial degradation may have affected it. A third possibility is that kallikrein is expressed in some, but not all, vascular territories.

The physiological role of the vascular kallikrein-kinin system is unclear. Although our data suggest that kallikrein is synthesized by vascular smooth muscle, we cannot overlook the possibility that it is also synthesized by endothelial cells. We do not know whether resistance vessels likewise synthesize kallikrein or whether vascular kallikrein is released and, if so, whether it remains within the vascular wall or passes into the circulation. Also, the presence of mRNA does not clarify whether the mechanisms for converting prokallikrein to active kallikrein exist in vascular tissue. However, preliminary data indicate that active kallikrein is released from rat vascular tissue in vitro (H. Nolly et al, Henry Ford Hospital, Detroit, Mich., unpublished data). Thus, the presence of a vascular kallikrein-kinin system, coupled with the aforementioned evidence, 13-17 suggests that local generation of kinins (autocrine/paracrine system) may contribute to regulation of vascular homeostasis.

In conclusion, we have demonstrated that kallikrein mRNA is present at low levels in rat arteries and veins, suggesting that it is synthesized by these tissues. We also found that vascular smooth muscle cells in culture contain kallikrein mRNA, suggesting that part of the kallikrein in the vascular wall originates from these cells. We propose that circulatory homeostasis may be regulated in part by generation of kinins within the vascular wall.

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